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The Effects of Various Therapeutics on Cystine Stone Formation

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The Effects of Various Therapeutics on Cystine Stone Formation

By

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A culminating thesis submitted to the faculty of Dominican University of California in partial fulfillment of the requirements for the degree of Master of Science in Biology

San Rafael, California

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CERTIFICATION OF APPROVAL

This thesis, written under the direction of candidate's thesis advisor and approved by the thesis committee and the MS Biology program director, has been presented and accepted by the Department of Natural Sciences and Mathematics in partial fulfillment of the requirements for the degree of Master of Science in Biology at Dominican University of California. The written content presented in this work represents the work of the candidate alone.

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TABLE OF CONTENTS

ABBREVIATIONS

TIO – Tiopronin

KCIT – Potassium Citrate

SWL - Extracorporeal Shock Wave Lithotripsy

PCNL - Percutaneous Nephrolithotomy

ARE – Antioxidant Response Element Pathway

LA - α Lipoic Acid

LIST OF FIGURES

ABSTRACT

Cystinuria is an autosomal recessive genetic disorder characterized by the defect of a renal transporter involved in cystine reabsorption. When this transporter is deficient, cystine cannot be broken down and reabsorbed by the body and is excreted via urine in high concentrations. The high levels of cystine present in the urine eventually lead to recurrent cystine urolithiasis due to its inability to solubilize. Despite having various forms of treatments such as thiol pharmaceutical therapies such as tiopronin and urine alkalinizing agents like potassium citrate, only few patients with cystinuria are able to successfully decrease cystine urine concentration. We observed the effects of tiopronin on its ability to inhibit and prevent stone formation and found it to be only moderately effective. In addition to observing tiopronin as a therapeutic, we also looked into the effects of potassium citrate as a treatment. We found potassium citrate to have a varying effects on both urinary pH and stone formation. Recently, our lab had shown that the nutritional-supplement α lipoic acid was an effective inhibitor of cystine stone formation. Here, we continued to further confirm the effects of α lipoic acid as an inhibitor and preventer of stone growth. Due to the unknown mechanism of α lipoic acid, we hypothesized α lipoic acid to be promoting cystine reabsorption through the Nrf2 pathway. In our *Slc3a1*$^{-/-}$*; Nrf2*$^{-/-}$ mouse model, the mice treated with α lipoic acid still continued to have a reduced rate of stone formation. These results suggested Nrf2 was not the pathway in which α lipoic acid was inhibiting stone growth. In addition, we observed the effects of the

combination of potassium citrate and α lipoic acid since potassium citrate is a standard of care of cystinuric patients. Our results suggested there are no adverse effects when the two drugs are administered together.

HIGHLIGHTS

- The commonly used thiol therapeutic for cystinuria patients, tiopronin (TIO), was shown to significantly decrease the rate of stone growth in the *Slc3a1*$^{-/-}$ mouse model. However, the therapeutic was observed not to be an effective stone formation preventer.
- Potassium citrate (KCIT), an alkalinizing agent and a therapeutic that is a part of the standard of care for all cystinuric patients, had varying effects on stone formation and did not successfully alkalinize urinary pH.
- Zee et al. recently reported the thiol antioxidant, α lipoic acid, to be an effective inhibitor of cystine stone growth and inhibition. Our results further confirmed this finding.
- Nrf2, a transcription factor that mediates antioxidant response element genes, was the hypothesized pathway α lipoic acid worked through to inhibit stone growth. However, results with the *Slc3a1*$^{-/-}$*; Nrf2*$^{-/-}$ mouse model indicate this was not the mechanism LA worked through to induce cystine stone inhibition in the *Slc3a1*$^{-/-}$ mouse.
- The administration of potassium citrate and α lipoic acid in conjunction was shown to have no adverse or additive or synergistic effects on one another

THESIS OVERVIEW

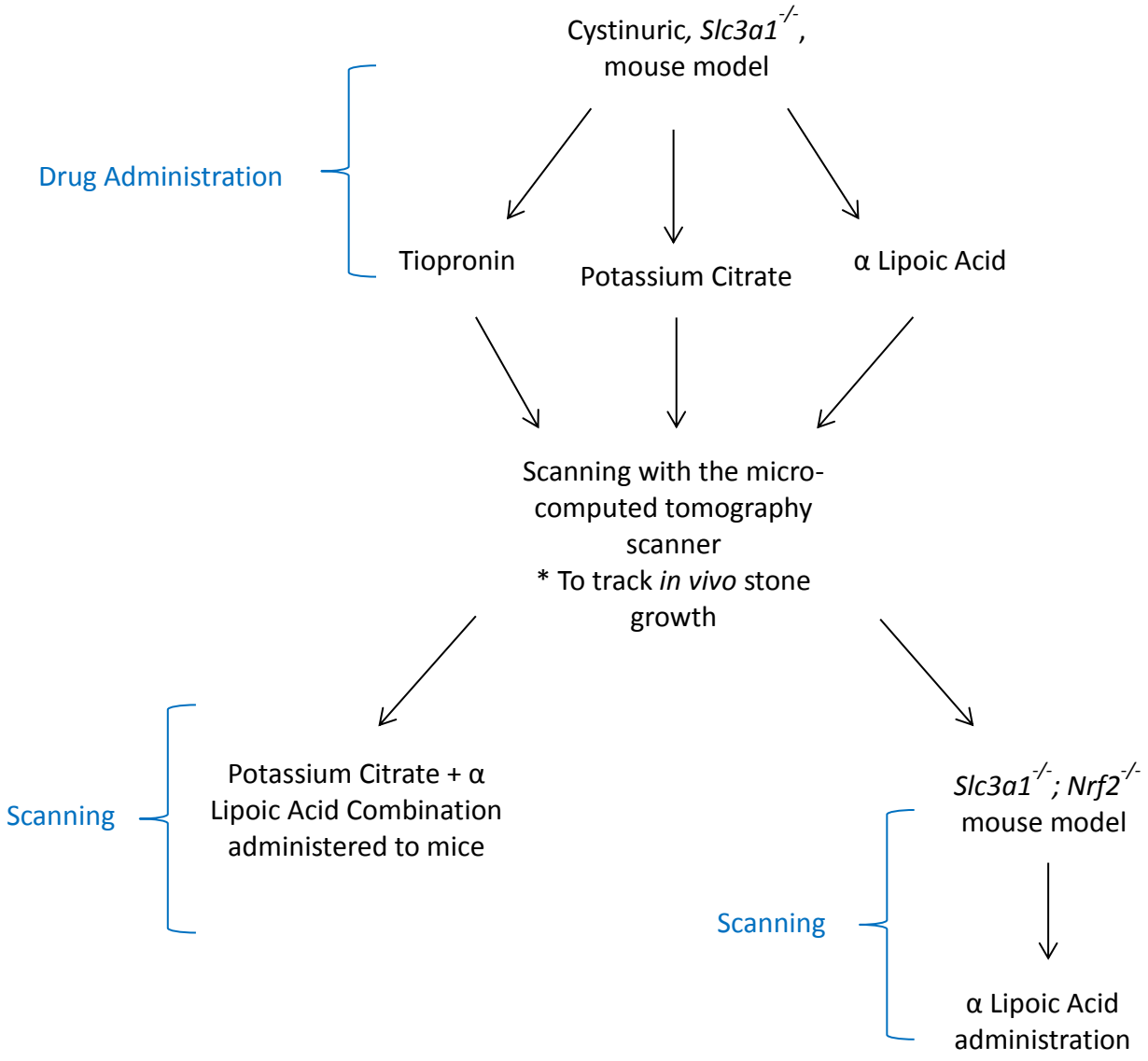

Fig. 1 – *Thesis overview.* Three therapeutics: tiopronin, potassium citrate, and α lipoic acid were administered to the cystinuric, *Slc3a1*$^{-/-}$ mouse model. The mice were scanned on a weekly basis in order to track *in vivo* stone growth. The combination of potassium citrate and α lipoic acid were administered to observe the effects of the two drugs may have on one another. A *Slc3a1*$^{-/-}$*; Nrf2*$^{-/-}$ mouse model was administered α lipoic acid to observe the role the Nrf2 pathway played in α lipoic acid's ability to induce stone rate inhibition.

BACKGROUND

Kidney Stone Disease is an Increasing Public Health Problem

Over the last 25 years, the prevalence of kidney stone formation has risen in the U.S. and affects approximately 9% of adult American regardless of sex or ethnicity (Arumuham & Bycroft, 2016; Scales, Smith, Hanley, & Saigal, 2012). This rise has been speculated to be caused by change of active lifestyles to sedentary ones and diet. Risk factors of stone formation generally include obesity, diabetes, and certain metabolic syndromes such as gout. Stone formers are at risk for hypertension, chronic kidney disease, and end-stage renal disease (Khan et al., 2016). Although males have traditionally had higher incidences of stone cases, recently females have been observed to have increased prevalence of stone formation. The possible reason behind this rise in stone incidences in females is the increasing obesity rates amongst both males and females (Arumuham & Bycroft, 2016; Khan et al., 2016). There are five types of kidney stones. Calcium oxalate is the most common stone type making up 80% of cases meanwhile other forms of stones such as calcium phosphate, uric acid, struvite, and cystine are respectively 5%, 9%, 8%, and 1% of stone incidences.

Idiopathic hypercalciuria patients make up 50% of calcium stone formers. This is a medical condition in which there is an increased excretion of calcium present in the body despite the lack of any diseases that could play a role in these elevated levels. There are certain systemic diseases such as primary

hyperparathyroidism and renal tubular acidosis that contributes to abnormal levels of calcium present in the blood. These conditions can lead or cause the patient to be at risk for calcium stone formation, but the majority of calcium based stone formers are idiopathic hypercalciuria patients (Coe, Worcester, & Evan, 2016; Khan et al., 2016; Worcester & Coe, 2008). Generally, patients with hypercalciuria excrete approximately over 300 mg of calcium per day (Lewandowski & Rodgers, 2004; Worcester & Coe, 2008). Normal patients excrete 100-250 mg of calcium per day depending on the diet of the patient. These increased levels of excretion can cause calcium based stones to arise. Although calcium oxalate stones accounts for 80% of stone formers, calcium phosphate stone incidences are generally also a small component of calcium oxalate stone formation and the incidences are accounted in only 5% of kidney stone forming patients.

Most uric acid stone formers either have gout or have a high protein diet paired up with low fluid intake and makes up 9% of stone formers. There is a high output of uric acid in these patients and this is either caused by the over production of endogenous uric acid, which occurs in medical conditions such as gout when uric acid cannot be properly metabolized, or there is a high dietary intake of purine-rich foods (Arumuham & Bycroft, 2016). The high levels of uric acid in the urine are insoluble in the concomitant acidic urine (pH level ≤ 5.5) that these patients excrete. Due to this insolubility, uric acid stone formation can arise.

Struvite stones, on the other hand, are observed in only 8% of stone formation cases and are potentiated by the bacterial infection of the *Proteus* or *Klebsiella* or *Providencia* species that hydrolyzes urea to ammonium (Goel & Wasserstein, 2012; Khan et al., 2016). The presence of ammonium raises the urine pH from neutral to alkaline values. The precipitation of struvite occurs in urine with these alkalinized conditions. Struvite stone formation can grow at a rapid rate into large stones.

Cystinuria is an Inherited Kidney Stone Disease

Cystine based stones are the least prevalent form of stones and make up only 1% of stone incidences. This type of stone case is usually the result of cystinuria, which is a genetically inherited kidney stone disease, caused by mutations in either or both SLC3A1 and SLC7A9 genes. These mutations specifically impair the heterodimeric cystine/dibasic amino acid transporters in epithelial proximal tubular cells of kidney nephrons (Mattoo & Goldfarb, 2008) (Fig. 2A). These impaired transporters lead to the oversaturation of cystine in the urine due to the lack of cystine reabsorption (Fig. 2B). Cystine is not soluble at the physiological pH of urine and this is the cause of cystine urolithiasis in patients with this disorder (Claes & Jackson, 2012).

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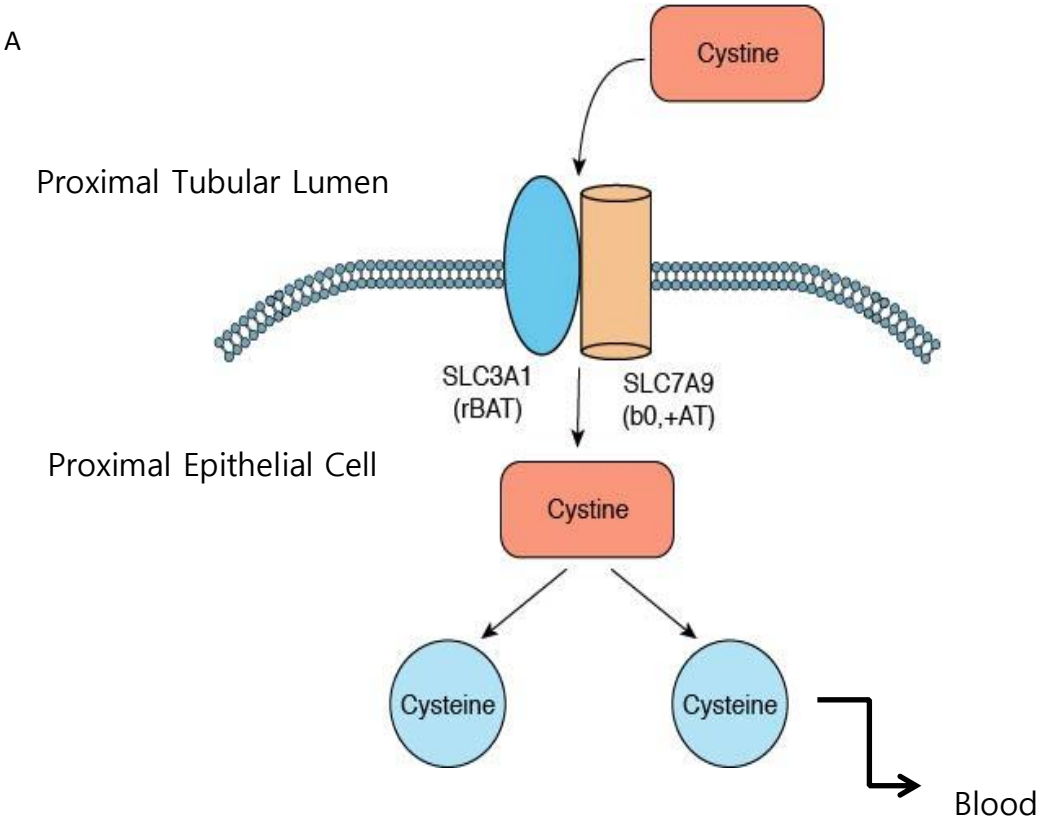
Cystine
Proximal Tubular Lumen
SLC3A1
(rBAT)
SLC7A9
(b0,+AT)
Proximal Epithelial Cell
Cystine
Cysteine
Cysteine
Blood

B

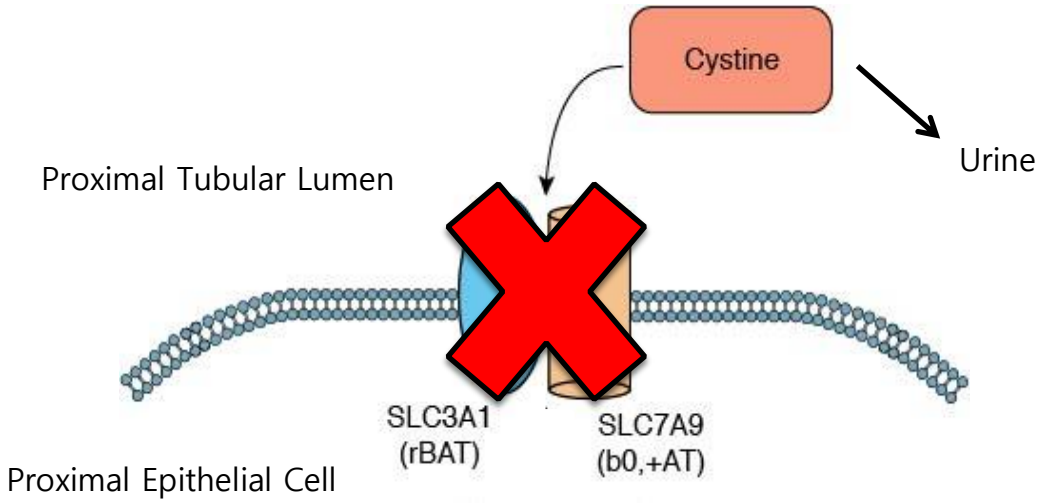

Fig. 2 – *Cystine reabsorption and the cystine transporter in normal patients and cystinuric patients.* The cystine transporter is a heterodimer of two subunits encoded by the genes SLC3A1 and SLC7A9 and is expressed on proximal epithelial cells. (A) In a normal patient, cystine is shuttled across the cystine transporter and is broken down into two cysteines. The cyteines are reabsorbed back into the body via the blood. (B) In cystinuric patients, the cystine transporter is defected due to a mutation in either the SLC3A1 or SLC7A9 genes. Thus, cystine cannot be broken down and is excreted via urine therefore there are elevated levels of cystine present in the urine of cystinuric patients.

Patients with cystinuria typically form their first kidney stone in the first two decades of their lives and continue to have recurrent stone formation, affecting their quality of life (Lee, Sahota, Ward, & Goldfarb, 2015; Varda et al., 2016). Due to the high rate of cystine stone formation, patients are required to have surgical interventions along with their oral therapeutics. Extracorporeal shock wave lithotripsy (SWL) is a minimally invasive way to surgically remove kidney stones by using high-energy acoustic waves to travel through the body to fragment kidney stones into smaller pieces that will be able to pass through the urinary tract. This is the preferred method of stone removal for most of the stone cases previously discussed. However, SWL isn't always the standard of stone removal for cystine based stones. Although SWL can be an effective treatment for cystine based stones, the composition of these types of stones are generally resistant to the acoustic waves and are not easily fragmented. Thus, multiple SWL treatments are necessary in order to fully fragment cystine stones (Claes & Jackson, 2012; Varda et al., 2016). The gold standard of care for cystine stone removal is percutaneous nephrolithotomy (PCNL), in which an endoscope is percutaneously directed through the skin, muscle, and fat into the kidney for stone removal and lithotripsy.

Treatment Options for Cystinuria are Limited

Patients with cystinuria excrete 300-400mg/L of cystine per day when normal patients only excrete 30 mg/L of cystine on a daily basis (Claes &

Jackson, 2012). In order to decrease this urine cystine concentration, patients are recommended to drink 3 to 4 L of water/day. With this method, a large consumption of fluid is necessary around the clock in order to keep the urine cystine concentration diluted. However, many patients find it difficult to maintain this method of treatment.

In the 1960s, Dent et al. observed cystine solubility to be pH dependent and this proposed another way to target cystine urine concentration other than increasing fluid intake. Urinary pH at 7.5 or above increases cystine solubility (Goel & Wasserstein, 2012). Thus, urinary alkalinizing agents such as potassium citrate, sodium citrate, and sodium bicarbonate are another form of treatment for cystinurics. Potassium citrate (KCIT) is the first line of alkalinizing agent that is often prescribed for patients and is also part of the standard of care for all kidney stone patients. For cystinuria, KCIT is the preferred form of treatment as compared to sodium citrate due to sodium's ability to increase cystine excretion in the urine. Although KCIT is a commonly used form of treatment for cystinuria and other stone models, there is currently no knowledge on this treatment's effect on stone growth. It is important to establish an understanding since KCIT is a widely used therapeutic for many kidney stone models.

Another method of increasing cystine solubility would be the use of pharmacological cystine-binding thiol medications. D-penicillamine and tiopronin (TIO) are two commonly used thiol drugs. The mechanism of how these drugs work is that they reduce the disulfide bond of cystine and the drug itself binds to one of the cysteine to make a drug-cystine complex. This complex

is presumably 50 times more soluble in urine than cystine on its own (Dolin, Asplin, Flagel, Grasso, & Goldfarb, 2005; Joly et al., 1999; Mattoo & Goldfarb, 2008). However, side effects of the thiol medications range from change in taste perception, rashes, fever, and proteinuria (Claes & Jackson, 2012; Halperin, Thier, & Rosenberg, 1981; Mattoo & Goldfarb, 2008). About 50% of patients experience side effects and long term therapy may lead to vitamin B6 deficiency, especially in those that are prescribed D-penicillamine. Thus, vitamin B6 supplements may also be prescribed in combination with the previously discussed treatments for patients that are using that particular thiol drug. (Biyani & Cartledge, 2006). Due to the adverse effects of D-penicillamine, TIO is the preferred form of treatment in the United States due to its lessened side effects and is more well-tolerated in patients (Claes & Jackson, 2012; Halperin et al., 1981; Joly et al., 1999; Mattoo & Goldfarb, 2008). Regardless of these lessened side effects of TIO, this drug can still have significant adverse effects on patients and this can lead to the discontinuation of usage.

The goal of these limited amounts of therapeutics available for patients mostly targets the oversaturation of cystine present in the urine to control or inhibit stone formation. It is clinically successful when the cystine urine concentration is less than 300mg/L, but only 15% of patients successfully achieve lower cystine concentration in their urine due to the inefficiency of these treatments (Mattoo & Goldfarb, 2008). Thus, there is a need to find other forms of therapeutics.

α Lipoic Acid as a Cystine Stone Inhibitor in the Mouse

α lipoic acid (LA) is commonly known as a thiol antioxidant of the organosulfur compounds family that was discovered as an effective stone inhibitor by our lab (Zee et al., 2017). As a therapeutic, LA was an effective inhibitor and preventer of cystine stone growth compared to control groups that were not administered the treatment in the *Slc3a1*$^{-/-}$ mouse model. Here, we repeated previous experiments and saw similar results of LA's ability to be an effective stone inhibitor and preventer.

On the other hand, as an antioxidant, LA play a role in the antioxidant response element (ARE) pathway against oxidative stress and various studies have shown the organosulfur compounds family had shown to upregulate Nrf2 activation (Cui et al., 2012; Lii et al., 2010). Nrf2 is a transcription factor regulating ARE-mediated genes. We hypothesized the Nrf2 pathway to be the mechanism in which LA was working through in order to induce stone inhibition and prevention by increasing Nrf2 nuclear translocation and cellular cystine utilization in order to inhibit cystine stone growth (Han et al., 1997; Suh et al., 2004).

Thus, one of the aims of our study focused on determining whether LA was working through the Nrf2 pathway and observe the effects of TIO and KCIT as a therapeutic for inhibiting stone growth and accumulation. In our study, we used the *Slc3a1*$^{-/-}$ mouse model which exhibit many symptoms similar to its human cystinuric counter partner and develop cystine urolithiasis. The micro-

computerized tomography scanner (μCT) was utilized to track stone growth and stone volume accumulated in our mouse model.

METHODS AND MATERIALS

Mouse Model

All procedures and protocols were approved by the Institutional Animal Care and Use Committee of the Buck Institute for Research on Aging. The mouse model used was the *Slc3a1*$^{-/-}$ mouse with an A129 and C57Bl/J6 mixed background. This model mimics all the symptoms human cystinuric patients present with the oversaturation of cystine in urine and the formation of cystine stones (Ercolani et al., 2010). Stone formation generally began around 2-3 months old. Only male mice were experimented on. Female mice do not generally form stones and are excluded from this study. Mice were kept in 12 hour light and 12 hour dark cycle at 24°C and had free access to food. An average number of 4 to 6 males are used for each treatment group.

Drug Treatments

A solution of potassium citrate tribasic monohydrate (Sigma Aldrich, St. Louis, MO) was mixed, changed, and weighed on a weekly basis to ensure the efficiency of the drug and measure whether or not the mice were drinking the solutions. The different dosages of 0.5%, 2%, and 4% KCIT solution were decided on previous KCIT studies on rat and other mouse models of other kidney stone diseases (Cebotaru et al., 2005; Krieger et al., 2015). The two other dosages, 1% and 1.5% KCIT, were decided later in the study as two possible effective dosages due to side effects of the 2% and 4% KCIT dosages. Mice were fed chow supplemented

with 0.25% LA or 0.5% LA or 1% tiopronin (Envigo, Huntingdon, United Kingdom). The control mice received normal water and chow. All mice involved in experiments were randomized prior their placements in treatment groups to avoid bias.

Urine Collection

Fresh urine was collected every 2 to 3 days. Mice were placed on cardboards covered with plastic wrap. Any collectable amounts of urine (20+ µL) present on the plastic wrap were collected immediately into Eppendorf tubes.

pH Reading

The Jenco 6230 pH meter (Jenco, San Diego, CA) was used to ensure pH difference between the samples soon after the urine collections.

Micro-Computed Tomography *(µCT)*

The µCT scanner (SkyScan 1176, Bruker, Belgium) is a noninvasive method used for to detect and to track the rate of stone growth. All mice received the general anesthesia of 2 to 3% isoflourane before and during the scans and were placed in a supine position. The scanner was set at these following settings: Resolution = standard, image pixel size = 1000 x 668, averaging frames = 2, filter = AL 0.5 mm, X-ray voltage = 50 kV, anode current = 500 µA, rotation = 0.7, and exposure time 4 minutes. Scans were performed weekly to detect, track, and measure the rate of stone growth.

Scan Analysis

Scans were reconstructed as cross-sectional three dimensional (3D) images with the use of the software, NRecon (NRecon v1.6.9.8, Bruker-MircoCT, Belgium). The dynamic range on this program was set to -0.002 to 0.08 to minimize the background noise and ensure the consistency between the individual mouse scans. The reconstructed 3D images were then exported to the image analysis software, CT Analysis (CTAn v1.14, Bruker-MicroCT, Belgium). A threshold between 70 to 120 was chosen to obtain better contrast between the bladder wall and stones. We used the circular region of interests to focus specifically on the bladder region of these 3D images to detect and calculate the measurements of the amount of stones present on a weekly basis.

Statistical Analysis

Littermate mice were randomly assigned to a treatment or control group for each experiment. Means and standard errors were calculated in each treatment and control group. Two-sided Student's t-tests were used to determine statistical significance between groups.

RESULTS

Cystinuria is a genetic disorder that causes the lack of cystine reuptake. The two genes that play a role in this disorder is SLC3A1 or SLC7A9. When either of these genes is mutated, the cystine transporters expressed on the proximal epithelial cells are defected. This deficiency leads to the lack of cystine reabsorption by the body and cystine is excreted in elevated levels through urine instead. The presence of excessive cystine in the urine presents a problem due to cystine's lack of solubility in the bodily fluid. Current medical therapeutics such as tiopronin or D-penicillamine target cystine solubility, but is often an inefficient preventative strategy, leaving patients with limited therapeutic options. Thus, we observed tiopronin's effect on stone formation since it is the preferred form of thiol treatment in the United States due to its lessened adverse effects on patients (Matoo & Goldfarb, 2008). The *Slc3a1*$^{-/-}$ mouse model we utilized in our experiments were administered TIO at 2-3 months of age after they have already started to form stones in order to observe TIO's inhibitory effects on cysteine stone growth (Fig. 3A). The results suggest there was a significant decrease on the rate of stone growth when we compare the TIO treated group to the control group. In order to observe TIO as a preventative measure, the drug was administered to younger mice around 1-2 months of age that had not yet formed stones or had very little stone accumulation present in their bladders (Fig. 3B). Our results indicate that although TIO was able to significantly decrease the rate of stone growth, this therapeutic was not an effective inhibitor of stone formation.

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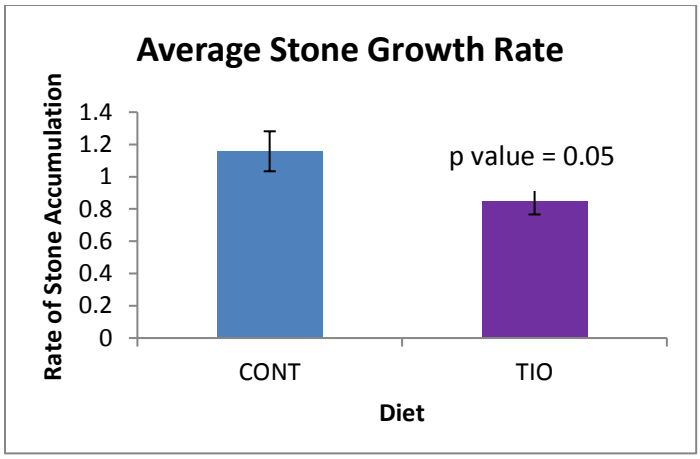

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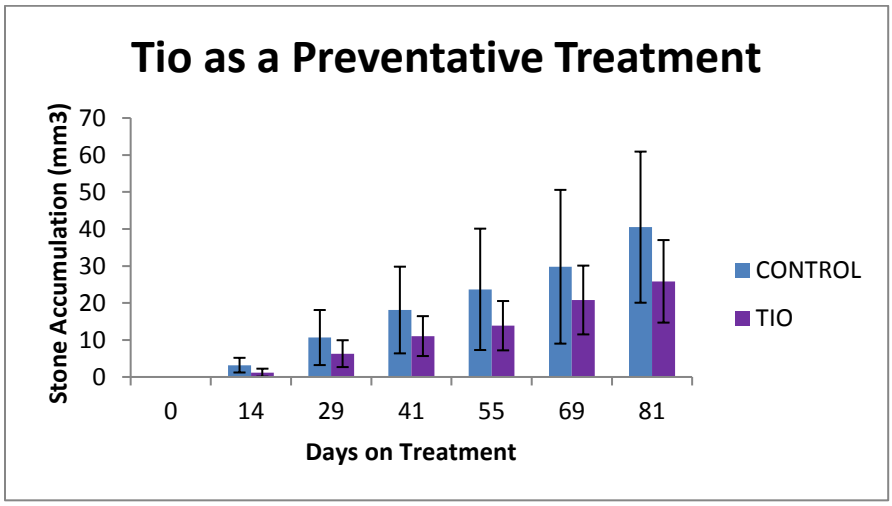

Fig. 3 –*TIO effects on stones as an intervening and preventative treatment on stone growth*. (A) 2-3 month *Slc3a1*$^{-/-}$ male old mice were administered normal (CONT) (n=8) chow and 1% TIO supplemented chow (n=9. Rates of stone accumulation were expressed as means ± SEM. (B) 1-2 month old male mice were administered CONT chow (n=9) and TIO supplemented food (n=9) and stone volume accumulated was tracked for 3 months. Stone volumes were expressed as means ± SEM.

In addition to observing tiopronin and its capabilities, KCIT's possible ability to inhibit stone formation was also looked into as well. Urinary pH is known to affect the solubility of cystine, thus urine alkalinizing agents such as KCIT is a commonly used mean of alkalinizing urinary pH as a standard of care for cystinuria patients. Although KCIT is commonly used for patients, there is

very little known about this alkalinizing agent and its capabilities in inhibiting the rate of stone formation. In our study, we want to use the highest effective dosage to look at the effects of KCIT on stone formation.

0.5%, 2.0%, and 4% KCIT solutions were administered to mice. Within the first week after the initial administration of the KCIT solution, the groups ingesting both the 2.0% and 4.0% KCIT solution were observed to have undergone either tremendous amounts of stress and were euthanized or were found dead. We immediately stopped the administration of the two highest dosages and began the administration of 1% and 1.5% KCIT solutions instead and with careful observations. Urine was collected on a 2-3 day basis and pH was measured by a pH meter. We discovered the 1.5% KCIT group eventually developed complications similar to the 2% KCIT and 4% KCIT groups, thus we discontinued the usage of it immediately. This action left 1% KCIT as our final and highest dosage.

We looked into the effects of KCIT on stone growth with a range dosages consisting of 0.25% KCIT, 0.5% KCIT, and 1% KCIT (Fig. 4A). Of the three various dosages we administered, 0.5% KCIT was the most efficient at inhibiting stone growth. Meanwhile the 0.25% KCIT and 1% KCIT had minimal to no inhibitory effect on the rate of stone growth. In addition to tracking these dosages' effects on stone growth, we also kept track on their effects on weight (Fig. 4B). All weights of all the groups were similar and within a normal range. In addition, the effect of KCIT on urinary pH was also observed (Fig.4C). Our results indicated that we were not successful in alkalinizing the urinary pH of our

mice. There were no statistical significance between the urinary pH levels of the untreated and treated mice.

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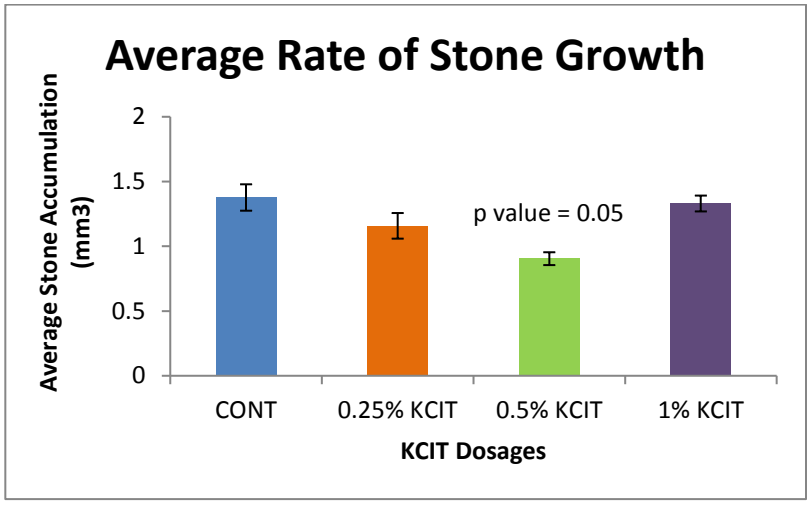

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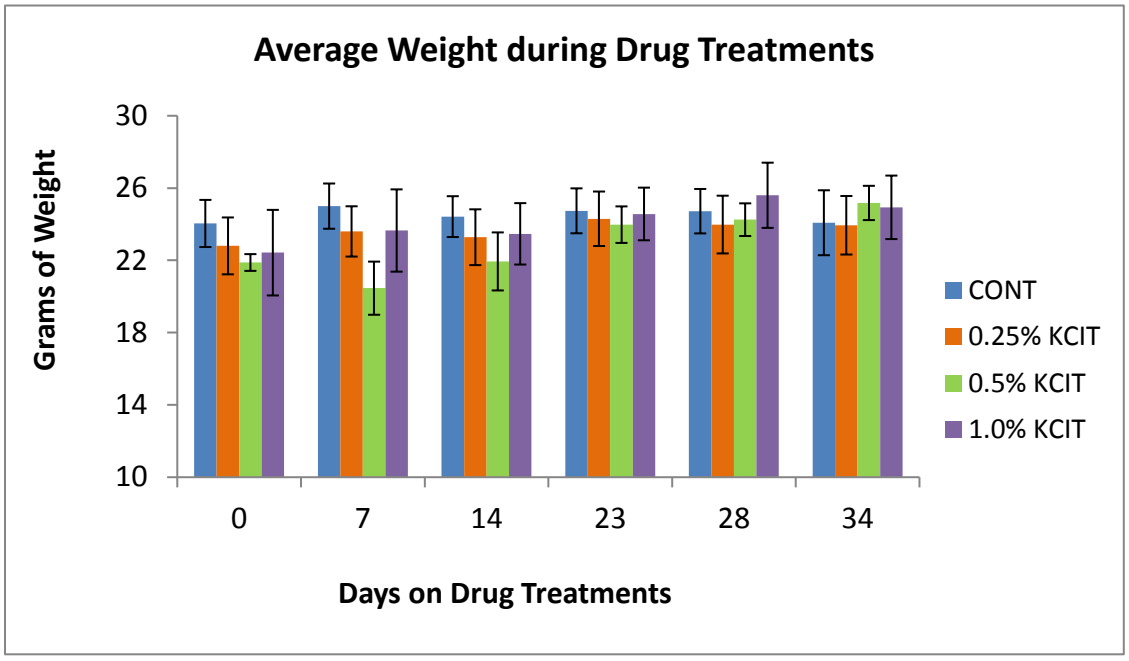

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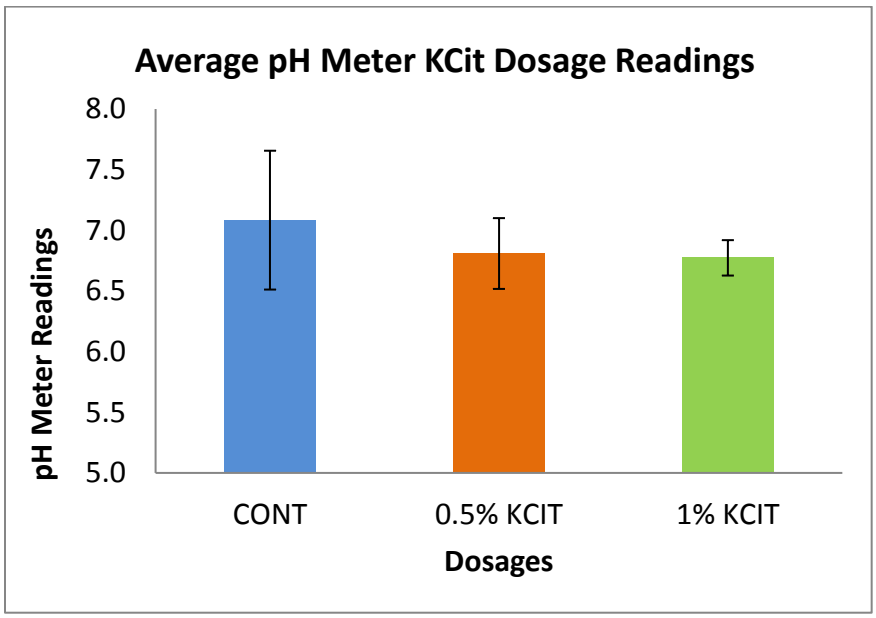

Fig. 4 – *Effect of varying dosages of KCIT on urinary stone formation, weight, and urinary pH on Slc3a1$^{-/-}$ mice.* (A) 2-3 month old male *Slc3a1$^{-/-}$* mice were administered three dosages ranging from 0.25% KCIT (n=5), 0.5% KCIT (n=5), 1% KCIT (n=4), and CONT water (n=5) for over a month; a total of 6 µCT scans were conducted. Rates of stone growth are expressed as mean ± SEM. (B) Weights of mice in the duration of the drug treatments. Weights are expressed as mean ± SEM. (C) 2 month old male *Slc3a1$^{-/-}$* mice were administered normal water, 0.5% potassium citrate (KCIT), or 1% KCIT solution for 2 months. Urine samples were collected on a 3-4 day basis. pH of urine samples was measured by a pH meter. The pH meter readings are expressed as mean ± SEM (n=4 per condition).

We recently reported the thiol antioxidant, LA, to be a successful inhibitor and preventer of cystine stone formation in the *Slc3a1$^{-/-}$* mouse mice (Zee et al, 2017). Here, we further confirm LA as an effective treatment of cystine stone formation and prevention. In order to observe the effects of LA as an inhibitory

drug, we administered the drug to mice around 2 to 3 months of age and tracked their rate of stone growth for 35 days (Fig. 5A). The group on LA treatment had a significantly lower rate of stone growth when compared to the control group. These results suggested that LA was a strong inhibiting treatment. In addition to observing LA as an inhibiting stone growth treatment, we also observed this treatment's capabilities as a preventative treatment. We administered LA to young mice that have not yet started to form stones or haven't accumulated much yet (Fig. 5B). Our results indicate LA to be an effective stone preventer of stone formation.

Since LA's mechanism of inhibiting stone formation is unknown, there are possibilities of it acting as a urine alkalinizing agent such as KCIT. Thus, urine from LA treated mice was collected to observe the possible alkalinizing effects LA may have on urinary pH (Fig. 5C). The results suggest that LA did not alkalinize the urinary pH of the mice during the administration of this drug. The possible mechanism in which LA may be working through in order to inhibit stone formation and prevention was not through alkalinizing urine. In addition, we also kept track of the weights of the mice in the duration of the LA administration to ensure the mice's weights were within a normal range and our results suggest that they were (Fig. 5D).

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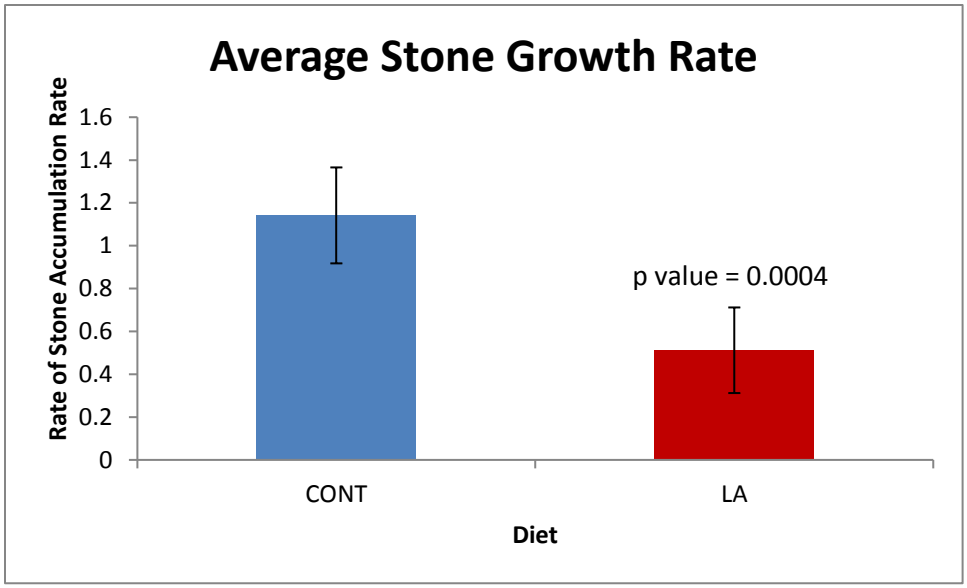
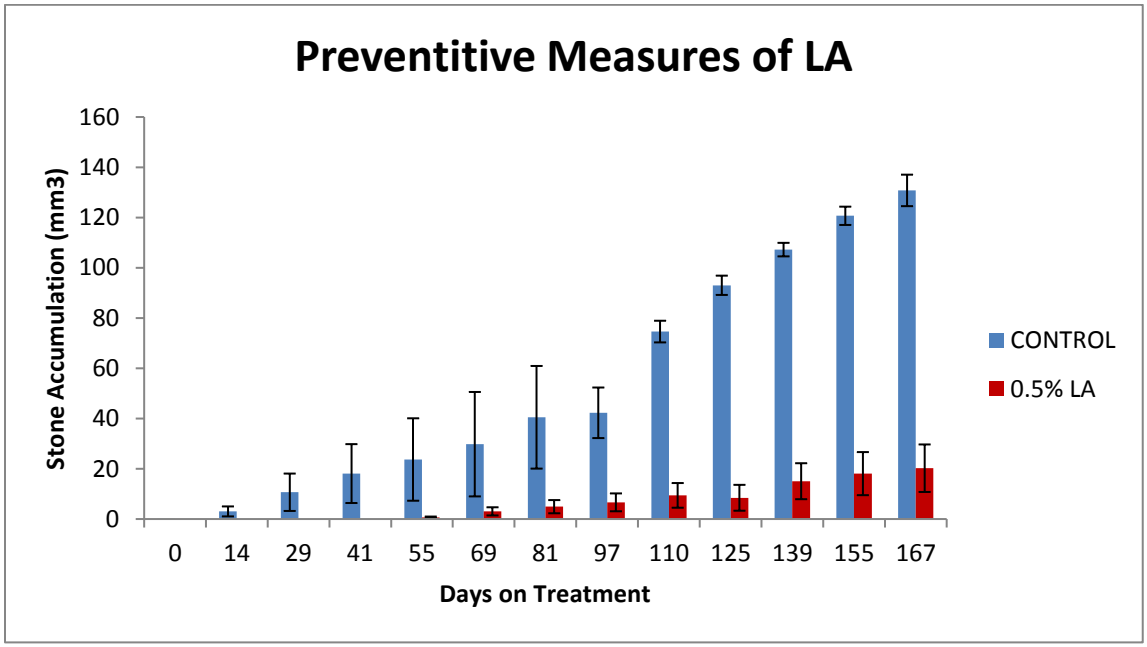
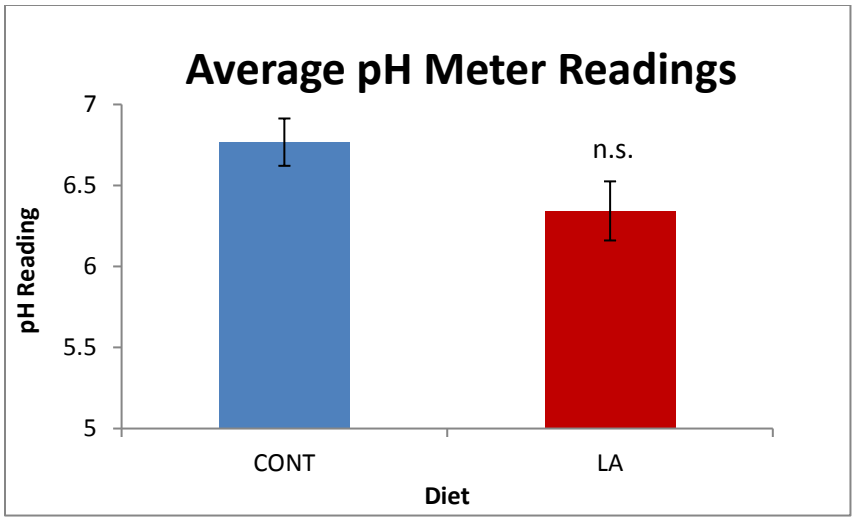

Average Stone Growth Rate
Rate of Stone Accumulation Rate
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
p value = 0.0004
CONT
LA
Diet

B


Preventitive Measures of LA
Stone Accumulation (mm3)
160
140
120
100
80
60
40
20
0
0
14
29
41
55
69
81
97
110
125
139
155
167
Days on Treatment
CONTROL
0.5% LA

C



D

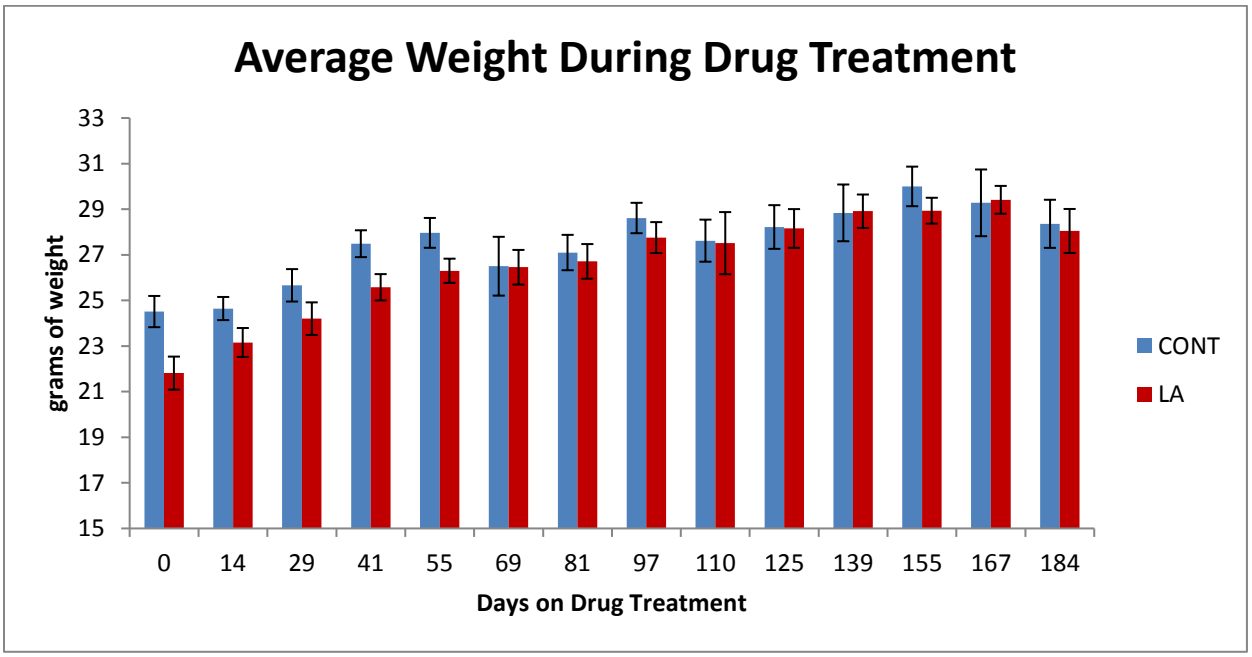
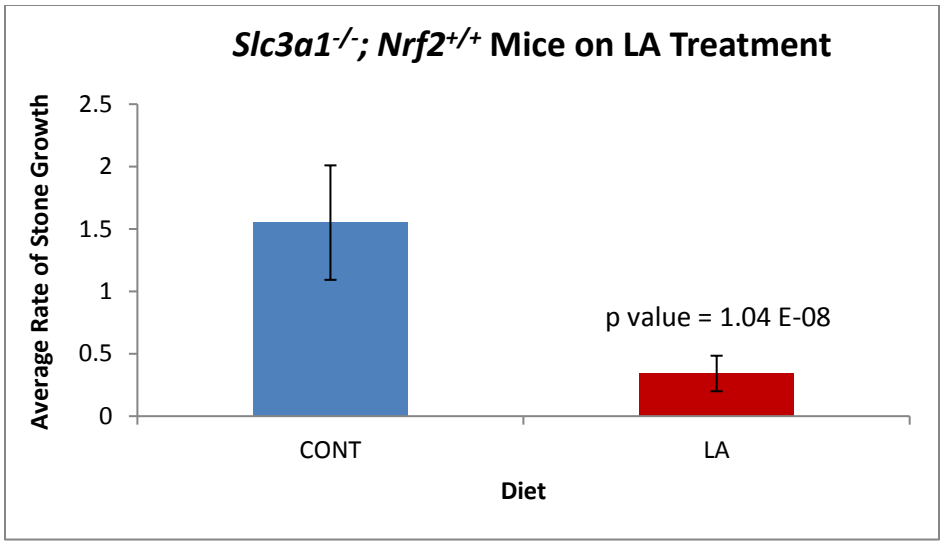


Fig. 5 – *LA as a treatment of cystine based stones*. (A) 2-3 month old mice were administered CONT food (n=8) and 0.5% LA supplemented chow (n=5) for over one month; a total of a total of 7 µCT scans were conducted. Results are expressed as means ± SEM. (B) CONT food (n=9) and LA supplemented food (n=9) was given to mice 1-2 month old mice and stone accumulation was tracked for 6 months. Results are expressed as mean ± SEM. (C) Urine samples were collected on a 3-4 day basis and pH values were measured via pH meter. Readings are expressed as mean ± SEM (n=5 per condition). (D) Weights of mice in the duration of the drug treatment. Weights are expressed as mean ± SEM (n=5 per condition). Two-tailed Student's tests were used to determine statistical significance.

The mechanism in which LA worked through in order to inhibit stone growth was unknown to us. As a known target of LA, the Nrf2 pathway was hypothesized to be the mechanism that LA worked through to inhibit stone growth. Nrf2 is an oxidative stress induced pathway that directly transcribes antioxidant and detoxifying enzymes for cellular defense (Suh et al, 2004; Shi et al, 2016; Shay et a, 2009). There are various studies that have shown organosulfur compounds to increase upregulation of Nrf2 activation (Cui et al., 2012; Lii et al., 2010). In the *Slc3a1*$^{-/-}$; *Nrf2*$^{+/+}$ mouse model, our results indicate LA was able to successfully inhibit stone growth as we had previously see when compared to the control group (Fig. 6A). We knocked out Nrf2 in our *Slc3a1*$^{-/-}$ mice to create a double knockout mouse model in order to observe the possible role the Nrf2 pathway had in LA's ability to inhibit stone growth (Fig. 6B). The results indicate that LA was still successful in inhibiting the rate of stone growth despite the knockout of Nrf2 in the *Slc3a1*$^{-/-}$ mouse. These results suggest that Nrf2 was not the mechanism in which LA worked through in order to inhibit stone growth.

A



B

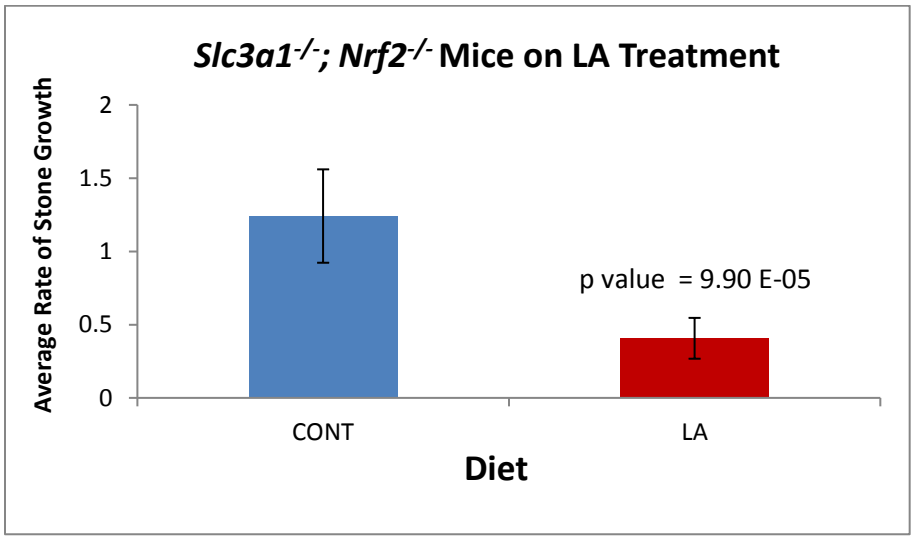
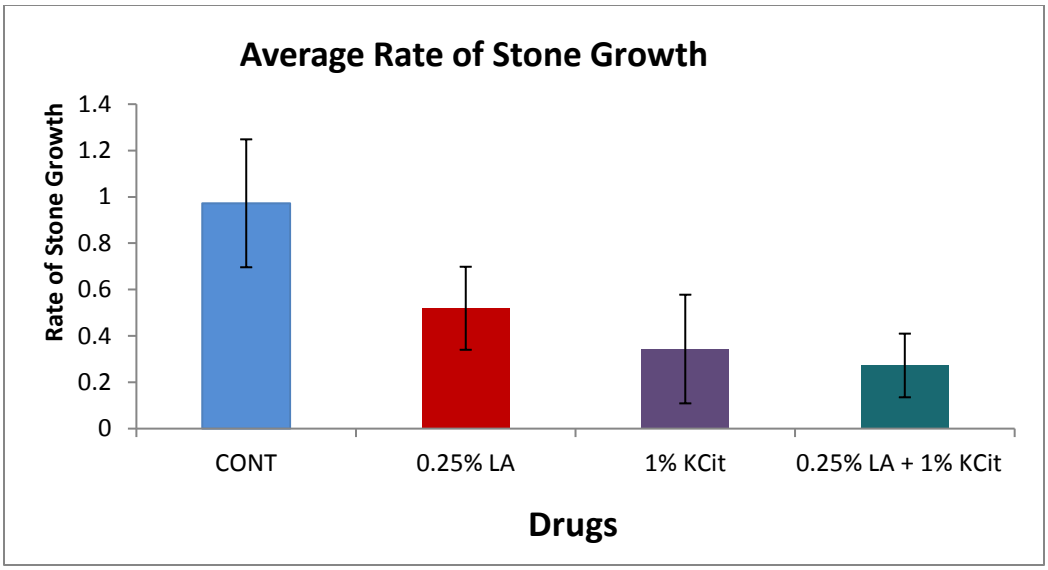


Fig 6 – *Nrf2 knockdown had no effect on LA's ability to inhibit stone growth.* (A) 2 month old *Slc3a1*$^{-/-}$*; Nrf2*$^{+/+}$ mice on LA treatment for 2 months; a total of a total of 11 µCT scans were conducted. Rate of stone growth are expressed as means ± SEM (n= 4 per condition). (B) 2 month old *Slc3a1*$^{-/-}$*; Nrf2*$^{-/-}$ mice on LA treatment for 2 months. Rate of stone growth are expressed as means ± SEM (n= 7 per condition). Two-tailed Student's tests were used to determine statistical significance.

Since urine alkalinizing agents are commonly used as a standard of care for patients (Goel and Wasserstein, 2012), we therefore addressed whether the usage of KCIT combined with LA would have an antagonistic, additive, or synergistic effects on stone inhibition. A low dosage of LA was selected to pair

up with a high dosage of KCIT. Our results showed that both the low dosage of LA and high dosage of KCIT was able to independently decrease the rate of stone growth (Fig. 7A). However, when we administer the two drugs in conjunction, there seemed to be no antagonistic, additive, or synergistic effects of the two. There are no significant differences between the different drug treatment conditions. The effects of the drugs on the rate of stone growth was also mirrored in the average amount of stone volume accumulated (Fig. 7B).

A



B

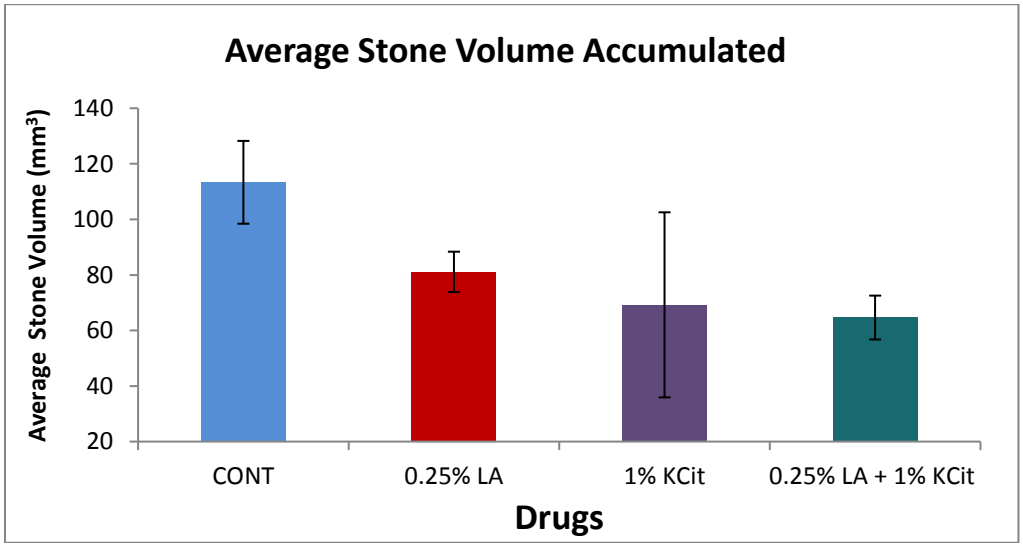


Fig. 7 – *Effect of KCIT and 0.25% LA on stone growth and accumulation.* (A) Average stone volume accumulation and (B) average rate of stone growth was tracked by the µCT scanner for 3 months; a total of a total of 9 µCT scans were conducted. The data are presented as means ± SEM (n=6 per condition). Two-tailed Student's tests were used to determine statistical significance.

DISCUSSIONS

Cystinuria is an autosomal recessive genetic disease that causes the lack of cysteine reabsorption through the mutation of one of the genes, SLC3A1 or SLC7A9. The mutation of either one of these two genes causes a defect in the cystine transporter present in epithelial proximal tubular cells and leads to the lack of cystine reabsorption. Since the body cannot reuptake cysteine due to the defect of this transporter, cystine is excreted in excess via urine instead. The oversaturation of cystine present in the urine eventually leads to cystine stone formation. There are currently no known pharmacotherapies that have been shown to successfully inhibit cystine stone formation although drugs such as D-penicillamine and tiopronin are commonly used (Claes & Jackson, 2012; Mattoo & Goldfarb, 2008). Aside from these thiol therapeutics, patients do not have much treatment options aside from using the urine alkalinizing agent and increasing fluid intake as methods to decrease aggressive cystine stone formation.

The conservative methods of treating cystinuria generally consist of the combination of increased consumption of fluids and using alkalinizing agents to target cystine urine concentration. These two methods are ineffective and the addition of thiol pharmaceutical drugs such as D-penicillamine and TIO are necessary to treat cystinuric patients. The use of D-penicillamine as a treatment began in 1963 when the drug was observed to have a significant effect on cystine solubility in patients of this genetic disease. Although the drug has been shown to have tremendous side effects on patients that includes rashes, fever, proteinuria,

and change in the perception of taste along with vitamin B6 deficiency since the 1960's, patients are still prescribed this drug treatment (Claes & Jackson, 2012; Crawhall, Scowen, & Watts, 1963; Halperin et al., 1981; Mattoo & Goldfarb, 2008).

Other forms of thiol therapies have been created since the introduction of D-penicillamine with the hope that they will have the same effect on cystine solubility with lessened side effects effects. Captopril and bucillamine are other thiol pharmacotherapies available aside from the previously discussed D-penicillamine and TIO. Unfortunately, there are few studies focused on these two drugs and the effectiveness of them. Captopril was shown to decrease cystine excretion in cystinuric patients, but the drug has been shown to have many inconsistencies and was suggested to be used for patients that cannot control their cystine stone formation with the use of D-penicillamine or tiopronin (Chow & Streem, 1996; Cohen, Streem, & Hall, 1995; Halperin et al., 1981; Sloand & Izzo, 1987). Meanwhile, bucillamine had only been previously available to patients in South Korea and Japan (Biyani & Cartledge, 2006). Thus, there a few available clinical studies in the United States that observes the efficacy of bucillamine as a new method of treatment.

TIO is currently the main choice of thiol treatment aside from D-penicillamine in the U.S. due to both the drugs' similar mechanism of action. Due to this similar mechanism of action, TIO also have a similar side effect profile as compared to D-penicillamine but this drug is better tolerated by patients. Both of these thiol drugs had been shown to be effective in decreasing

the rate of stone formation in patients, but many were still considered at a high risk of stone reoccurrence (Chow & Streem, 1996). Our results mirrored what has been seen in regards to TIO's modest capability to intervene in the rate of growth in our mice. Just as Chow and Streem had observed, our results indicate that TIO was unable to successfully prevent stone formation unlike LA as a treatment.

Zee et al reported the oral administration of the thiol antioxidant, LA, as an effective cystine stone inhibitor and preventer in the *Slc3a1*$^{-/-}$ mouse model as compared to untreated *Slc3a1*$^{-/-}$ mice. In this study, LA was identified as an effective inhibitor of stone growth through a drug screen consisting of various compounds that have been shown to activate the ARE pathway to promote glutathione synthesis and increase cellular cysteine uptake. Our results further confirmed the report of LA's abilities to act as an efficient inhibitor and preventer of cystine stone formation. LA is a known thiol antioxidant that has been shown to increase Nrf2, a transcription factor that mediates ARE mediated genes, translocation and increase cellular cysteine utilization (Cui et al., 2012; Lii et al., 2010). Due to these previously shown effects of LA, Nrf2 was the pathway we hypothesized LA worked through in order to induce stone inhibition and prevention in our mice. However, our results indicated that LA worked independently of the Nrf2 pathway to inhibit stone formation in our *Slc3a1*$^{-/-}$*; Nrf2*$^{-/-}$ mice. The effects of LA treatment on the rate of stone growth in *Slc3a1*$^{-/-}$*; Nrf2*$^{-/-}$ mice were not significantly different compared to *Slc3a1*$^{-/-}$ mice.

Alkalinizing agents is a standard of care for all cystinuric patients in order to increase urinary pH and increase cystine solubility (Dent, Friedman, Green, &

Watson, 1965; Joly et al., 1999; Mattoo & Goldfarb, 2008). KCIT is the preferred form of alkalinizing agents for patients over others forms. Citrate treatments such as sodium citrate increase the cystine excretion in patients and this is not ideal in the clinical setting. The increased excretion of cystine in the urine may have an impact on the rate of stone reoccurrence in cystinuric patients thus sodium citrate is a second line of treatment if necessary. However, the usage of KCIT and increasing liquid intake are generally not enough to tackle stone formation and prevention alone. Thus, the addition of thiol drugs is necessary (Barbey et al., 2000; Dolin et al., 2005). When we observe KCIT as a treatment without the addition of increased fluid intake or thiol drugs, the treatment was shown to have varying effects on stone formation and urinary pH. This may be due to various factors. In regards to urinary pH, a factor as to why KCIT was not successful in alkalinizing the urine of our mice may be due to KCIT's varying effects on urinary pH in the clinical setting and our results may be mirroring what is commonly seen in the clinic (Asplin & Asplin, 2013; Barbey et al., 2000). Or, another possibility is that KCIT may be working through another mean of action that is independent of urinary pH such as through its metabolites in order to increase cystine solubility and inhibit stone formation.

Some inconsistencies could be due to how KCIT was administered to our mice since the method of drug administration plays a crucial role in any experimental design (Turner, Brabb, Pekow, & Vasbinder, 2011). KCIT was given to the mice in their drinking water instead of other methods such as gavage or a KCIT supplemented diet to mirror clinical settings (Joly et al., 1999; Mattoo

& Goldfarb, 2008). It was observed that the water levels of the 1% KCIT solution were slightly higher than the other dosage solutions when water bottles were being changed out on a weekly basis. This could have been an indication that mice under the higher dosages of KCIT may have not been drinking as much water as mice on the lower dosages of KCIT or control water. These differences in water levels were not significant and did not raise much of a concern. However, we have taken into consideration that when a drug is administered through a solution, we cannot account for the proper dosage the mice are ingesting. In order to ensure the mice ingest the proper dosage in future experiments, gavage or the usage of a supplemented diet will be taken into consideration.

The treatments for cystinurics generally consist of the combinations of high fluid intake, urine alkalinizing agents, and thiol drugs. Although thiol drugs can significantly decrease stone formation and increase cystine solubility, there is still a high risk of rapid stone reoccurrence in patients (Barbey et al., 2000; Chow & Streem, 1996; Dolin et al., 2005; Khan et al., 2016). Thus, many patients are required to have close surveillance from physicians such as frequent visits to the clinic to perform radiological imaging and 24 hour urine collections. As a method to target urine cystine solubility, KCIT is the standard of care for all cystinuric patients. When LA is administered in the clinical setting, this new drug treatment will be administered in combination with KCIT. Both KCIT and LA were administered in conjunction to observe the possible adverse, additive, or synergistic effects the two drugs have on one another. There are many questions

that can arise in regards to why a high dosage of KCIT was paired with a medium dosage of LA. A high dosage of KCIT was chosen for the combination experiment due to our interest in mirroring clinical settings. For the clinical setting, patients will be administered the highest tolerable dosage for their specific body mass indexes. As discussed before, the 1% KCIT was discovered to be the most tolerable dosage for our mice without any detrimental side effects through a dosage experiment with the higher dosages of 1.5% KCIT, 2% KCIT, and 4% KCIT. There are possibilities that this highest tolerable dosage of KCIT may be masking the possible additive or synergistic effects of the administration of KCIT and LA in conjunction. For future experiments, we shall consider administering a lower dosage of KCIT.

For future directions, we plan to look into the mechanism of how LA inhibits the rate of stone growth in our mice. In this particular study, we showed that LA worked independently of alkalinizing urinary pH and the Nrf2 pathway to inhibit stone growth and formation. Thus, the mechanism of LA to induce stone inhibition and prevention is still unclear. We currently hypothesize LA to be working through its metabolites to increase cystine solubility in the urine (Zee et al., 2017).

In addition, there is an interest to start observing the effects of a LA supplemented diet on other stone models aside from cystinuria and its effects on stone growth and prevention. The stone model of interest will be the calcium oxalate kidney stone model. This stone disease is the most commonly diagnosed stone model with a prevalence of 80% of kidney stone cases (Goel & Wasserstein,

2012; Khan et al., 2016). As discussed previously, KCIT is a standard of care for cystinurics due to its observed ability to increase cystine solubility through the means of increasing urinary pH. This alkalinizing agent is also a standard of care for calcium stone formers because of citrate's ability to bind to calcium and reduce the amount of calcium that is available to bind with oxalate (Coe et al., 2016; Khan et al., 2016). In our future studies, we plan to observe the effects and efficiency of KCIT along with LA in the context of the calcium oxalate kidney stone model just as we had previously done with the cystinuric kidney stone model.

We will use the primary hypoxaluria type 1 disease model to observe KCIT and LA's effects on calcium oxalate stone formation. Primary hypoxaluria is a genetic disorder characterized by the mutation of the alanine-glyoxylate aminotransferase in the liver (Dutta et al., 2016). When this aminotransferase is defected, oxalate is overproduced by the liver and is present in the kidney and urine in abnormally high amounts. The high presence of oxalate leads to the crystallization and eventually formation of calcium oxalate based kidney stones. Although this disorder is hereditary, we want to use a model that will ensure the formation of calcium oxalate stones. Methods such as the administration of ethylene glycol to induce hyperoxaluria and hypercalciuria in mice may be inefficient and may take months before the initial formation of calcium based bladder stones. Thus, the primary hypoxaluria type 1 disease model will be our calcium oxalate stone model of interest and we will observe the effects of KCIT and LA on this type of stone formation.

CONCLUSIONS

In summary, our results showed that TIO had a modest effect both on stone growth inhibition and did not successfully prevent stone formation. In our study, the varying effects on urinary pH and stone growth with KCIT were consistent with what was seen in the clinical setting. We further confirm the efficiency of LA as a stone growth inhibitor and stone preventor (Fig. 1 and Fig. 3). We hypothesized the Nrf2 pathway to be the mechanism in which LA worked through in order ot inhibit the rate of stone growth. When LA was administered to the *Slc3a1*$^{-/-}$ *; Nrf2* $^{-/-}$ mouse model, our results indicated that Nrf2 was not the pathway LA worked through in order to inhibit stone growth. When we pair up KCIT with LA, there seemed to be no adverse effect on LA's ability to inhibit the rate of stone growth. This is an important observation for clinical settings when both KCIT and LA will be administered in combination with one another. Our research suggsted that LA is a new possible therapeutic for patients with cystinuria since it has been shown to be an effective intervening and preventative treatment.

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